IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Hisakazu KATSUKI et al
Application No.: 10/588,609
Filed: August 7, 2006
For: ED-71 PREPARATION

Confirmation No. 8366
Art Unit: 1616

Examiner: QAZI, SABIHA NAIM

DECLARATION

Honorable Commissioner of Patent and Trademarks
P.O.Box 1450
Alexandria, Virginia 22313-1450

Sir:

I, Hitoshi SAITO, a Japanese citizen, residing at 33-3 Fuji Village, Mishima-city, Shizuoka-prefecture, Japan, hereby solemnly and sincerely declare and state that:

I was awarded M.Sc. in 1989 from the Faculty of Science and Technology, Science University of Tokyo, Chiba-prefecture, Japan;

I have been employed by Chugai Pharmaceutical Co. Ltd., the assignee of the present application, since 1997, and worked at Fuji-Gotemba Research Laboratories in Gotemba, Shizuoka, Japan from 1997, as a researcher of bone biology field during the entire period.

I declare further that I engaged as a researcher in research into vitamin D3 preparations.

I declare further that I have read the Official Action in the above-identified application, and have read, and am familiar with each of the references cited in the Official Action by the Examiner.

Purpose of this declaration

The purpose of this declaration is to show experimental data to establish an advantageous effect of (5E,7E)-(1R,2R,3R)-2-(3-hydroxypropoxy)-9,10-secocholesta-5,7,10(19)-triene-1,3,25-triol (trans form of ED-71).

I declare that the following tests were conducted at my direction or under my supervision, and that the test results are true and correct to the best of my knowledge.

Materials and Test Method

1. Test Samples

Trans form of ED-71 (lot No.: YM99G045-4-1)

Site of manufacture: Chugai Pharmaceutical Co. Ltd, Synthetic Technology Research Laboratory

Supplier: Chugai Pharmaceutical Co. Ltd, Synthetic Technology Research Laboratory

Supplied form: ethanol solution (1mg/mL)

Storage conditions: in a light-shielding vial, in a freezer at a preset temperature of –20℃ or less

Control 1: 1α, 25-$(OH)_2$-D_3 (lot No.: WVC99AJ87)

Manufacturer: SOLVAY PHARMACEUTICALS

Supplier: Chugai Pharmaceutical Co. Ltd, Chemistry Research Laboratory

Supplied form: ethanol solution (1.02mg/mL)

Storage conditions: in a light-shielding vial, in a freezer at a preset temperature of –20℃ or less

Comments: This substance was selected to show an effect of a representative active vitamin D as a positive control, for comparison with trans form of ED-71.

Control 2: ED-71 (lot No.: 8G03ED)

Site of manufacture: Chugai Pharmaceutical Co. Ltd, Synthetic Technology Research Laboratory

Supplier: Chugai Pharmaceutical Co. Ltd, Synthetic Technology Research Laboratory

Supplied form: powder (25mg)

Storage conditions: in a light-shielding vial, in a freezer at a preset temperature of –20℃ or less

Used form: Before testing ED-71, a ED-71 solution in ethanol (0.93mg/mL)

was prepared, and stored in a light-shielding vial filled with argon gas, in a freezer at a preset temperature of –20℃ or less. Ethanol was selected since it is commonly used as a solvent for vitamin D derivatives.

Comments: ED-71 substance is a parent compound of trans form of ED-71.

2. Cells used

Cells: HL-60 cells

Subculture method: subculture was conducted in RPMI-1640 medium supplemented with 10% of fetal calf serum, at 37℃, in an atmosphere of 5% CO_2 in air.

Storage location: -130℃ freezer

Origin (animal species): human acute myelogenous leukemia cell strain (supplied by Chugai Pharmaceutical Co. Ltd, Pharmaceutical Technology Research Laboratory

3. Experimental Method

HL-60 cells were subcultured in RPMI-1640 medium supplemented with 10% of heat-inactivated fetal calf serum and 20μg/ml gentamycin, at 37℃, in a humidified atmosphere of 5% CO_2 in air.

An ability of induction of differentiation was estimated by the ability of HL-60 cells to generate a superoxide anion.

Each of the solutions of control samples (1α, 25-$(OH)_2$-D_3 and ED-71) and trans form of ED-71 (about 10^{-3} mg/mL) was diluted by use of 10 volumes of RPMI-1640 medium seven times sequentially, to produce sample solutions with a concentration of $1x10^{-10}$-$1x10^{-4}$mg/mL. HL-60 cells were seeded at $1x10^5$cells/mL in a growth media and cultured for 4 days in the presence of various concentrations of the sample solutions, to induce differentiation. Then, the cells were washed free of the compounds, and suspended in a 1.5mL reaction mixture containing 80μM ferricytochrome c (Sigma Chemical Co., St. Louis, MO; Sigma code: C-2506), and 500 ng/mL phorbol myristate acetate (Sigma; Sigma code: P-8139) in 0.1% gelatin Hank's balanced salt solution without phenol red. The mixture was incubated at 37℃ for 60 min, and centrifuged for 10 min. at 400xg at 4℃. The reduction of ferricytochrome c was measured by use of the absorption increase at 550 to 540 nm (molar absorption coefficient, $19.1x10^3$/cm) with a Hitachi U-3200 double-beam spectrometer. The results are shown in Figure 1 and Table 1 below.

Results

Fig 1 Differentiation-Inducing activity on HL-60 cells

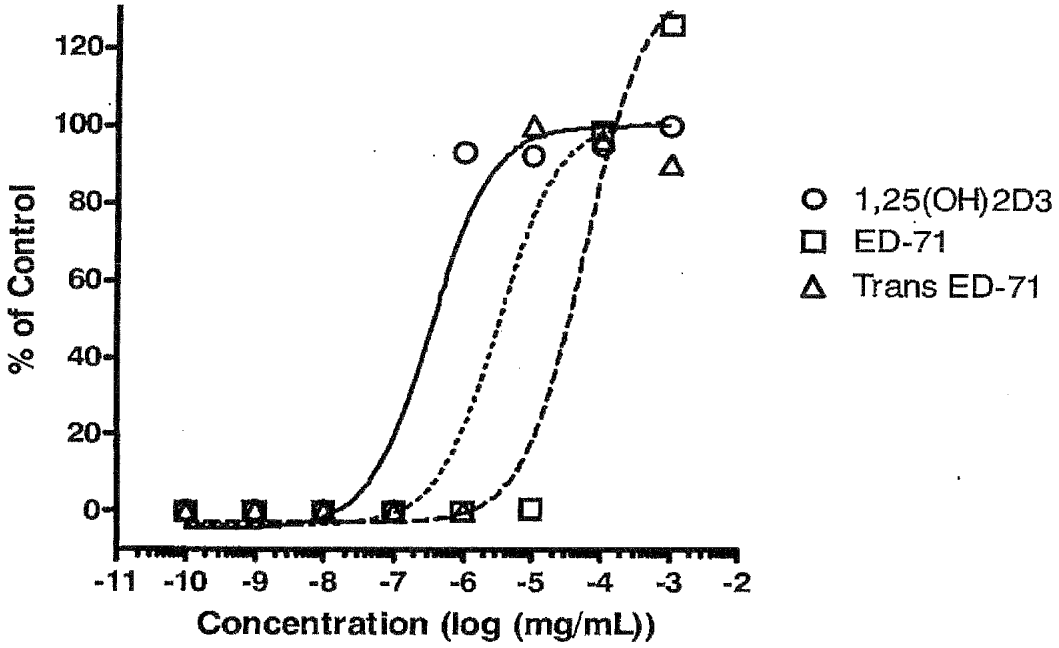

Table 1 Comparison of differentiation-inducing activity on HL-60 cells

Vitamin D derivatives	EC_{50}	Relative differentiation-inducing activity calculated based on 1α, 25-$(OH)_2$-D_3
1α, 25-$(OH)_2$-D_3	4.91×10^{-7}	1
ED-71	5.77×10^{-5}	0.0085
Trans form of ED-71	2.84×10^{-6}	0.1731

As shown in Table 1, trans form of ED-71 showed a relative differentiation-inducing activity on HL-60 cells of 0.1731 (this value was calculated from EC_{50} of the trans form, on the basis of EC_{50} of 1α, 25-$(OH)_2$-D_3, with regarding EC_{50} of 1α, 25-$(OH)_2$-D_3 as "1"). This value is almost 20 times higher than that of the parent compound, ED-71.

Conclusion

Trans form of ED-71 of the present invention shows a differentiation-inducing activity of almost 20 times higher than that of the parent compound, ED-71. I consider that this fact

supports an advantageous effect of the present invention beyond expectations of those skilled in the art. Therefore, I trust that the present invention of trans form of ED-71 is unobvious over the citations.

I declare further that all statements made therein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the instant patent application or any patent issuing thereon..

Dated this 29th day of January, 2008

Hitoshi SAITO